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ALKALINE PHOSPHATASE AND AN ACID ARYLAMIDASE AS MARKER ENZYMES FOR NORMAL AND TRANSFORMED WI-38 CELLS

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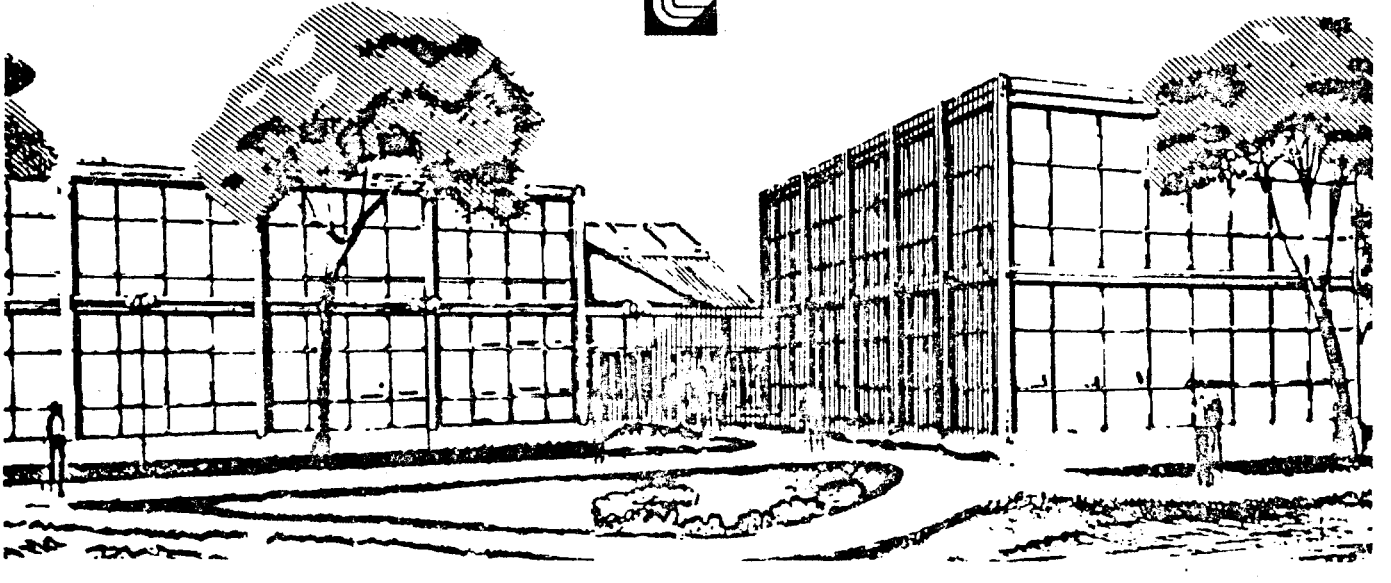

Marker Enzymes of WI-38 Cells

Alkaline Phosphatase and an Acid Arylamidase as Marker Enzymes for Normal and Transformed WI-38 Cells

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ABSTRACT

A survey of eleven enzyme activity levels in normal and SV40 transformed (VA-13) WI-38 cells revealed that the transformed cells differed from the normal cells by a quantitative and qualitative change of alkaline phosphatase and a quantitative loss of an arylamidase. Alkaline phosphatase activity was found to be elevated in the transformed cells at confluency but not in log phase cultures. This elevated activity was heat stable, L-homoarginine resistant and L-phenylalanine sensitive and is, thus, the term placental isoenzyme. In non-transformed WI-38 cells, the alkaline phosphatase was heat labile, L-homoarginine sensitive and L-phenylalanine resistant and so is the liver isoenzyme. While the arylamidase activity from both normal and transformed WI-38 cells had identical pH optima and Km values, the activity was approximately 20 times higher in confluent WI-38 cells than in confluent VA-13 cells. Cytochemical staining techniques for both activities are described which permit identification of fluorescent product within the cells, analysis of activity levels, and separation of cells with high and low activities. Mixtures of WI-38 cells and VA-13 cells separated by flow cytometry on the basis of arylamidase activity were subsequently evaluated for alkaline phosphatase isoenzyme and found to have been simultaneously separated into heat labile and heat stable samples.

INTRODUCTION

Quantitative cytochemistry of intact cells is a relatively new approach to studies in carcinogenesis that offers several advantages over the more established disciplines of biochemistry and cytology. Quantitative cytochemistry seeks to measure the biochemical properties of individual cells through a combination of specific probes and cell-oriented instrumentation. In this context we use the term "marker" to identify differences between cells (e.g., DNA content, enzymatic activity, and antigenicity) and "probes" to mean the methods used to visualize the markers (e.g., optically active DNA ligands, enzyme substrates and fluorescently tagged antibodies).

Numerous biochemical studies have indicated significant differences in the levels of various enzyme activities between normal and neoplastic cells. Elevated enzyme levels in the glycolytic and DNA synthetic pathways have been observed in experimental hepatomas (42). Serum acid phosphatase has been used for years as a marker for human prostate cancer (9). Several isoenzymes of alkaline phosphatase have been detected in sera from patients with various neoplasias (7). Sialyltransferase (13) and 5'-nucleotidase (35) frequently are elevated in cases with either primary or metastatic tumors (13). γ-Glutamyl transpeptidase, adenosine triphosphatase, glucose-6-phosphate dehydrogenase (5) have become markers for experimentally induced preneoplastic lesions in rat liver. A high level of plasminogen activator has been associated with cell transformation in vivo (27) and in vitro (28,38).

We have recently reported a fluorescent cytochemical method for detecting γ-glutamyl transpeptidase and its use in identifying

presumptive preneoplastic cells in rat liver cell suspensions by flow cytometry (39). We report here two other enzymes which serve as identifying markers of WI-38 cells and SV 40, VA-13 cells (8). We also report on fluorescent cytochemical methods which may be used for these marker enzymes and distinguish these cells cytofluorometrically. The rationale for developing fluorescent cytochemical probes and applying them to mixtures of "normal" and "transformed" cells is the expectation that these methods will have more general utility in automated cytology.

A preliminary report of these data has been published (3).

MATERIALS AND METHODS

WI-38 (11) and VA-13 cells (8) were obtained from the American Type Culture Collection and grown in Ham's medium MCDB103 (10) with 10% calf serum, (Kansas City Biological, Lanexa, Kansas). VA-13 cells are SV40 transformed WI-38 cells (8). WI-38 cells between passage 20 and 26 were passaged every fifth day with a split ratio of 1:3 and VA-13 cells were passaged every third day with a split ratio of 1:5. For enzyme assays, cell monolayers from 100 mm plastic dishes were washed three times with phosphate buffered saline, pH 7.4. The cells were scraped from the culture dishes, resuspended in 1 ml of 50mM Tris buffer, pH 7.5, and sonicated in an ice bath for 10s with a Branson Sonifier. Aliquots of sonicated homogenate equal to approximately 10^5 cells were used for individual assays.

The substrates and assay conditions are listed in Table I. Substrates containing 4-methoxy-2-naphthylamine (MNA) (Enzyme Systems Products, Indianapolis Indiana) were assayed by direct fluorometric analysis as described previously (4,37). Hydrolysis of substrates containing p-nitrophenol, p-nitroaniline, or p-nitrocatechol (Sigma Chemical Company, Saint Louis, Missouri) was measured by absorption at 410, 405, and 560nM respectively after the solutions had been raised to pH 10.5 with 0.5M sodium glycinate buffer. All assays reflect the extent of hydrolysis at 37^{o} for periods up to 1 hr and do not necessarily reflect initial velocities or optimal reaction conditions. Measurements were corrected for spontaneous substrate hydrolysis and turbidity. Protein content of cell homogenates was determined by the method of Lowry et al. (22).

Further studies were made on the alkaline phosphatase in crude homogenates of cells. The hydrolysis of 1mM naphthol-1-phosphate (Sigma Chemical Company) in 0.1M Tris-borate buffer, pH 10.2, with 5mM $MgCl_2$ was measured fluorometrically (λ ex=335nm, λ em=455nm). The effects of addition of L-homoarginine or L-phenylalanine, (A grade, Cal Biochem-Behring, San Diego) or of pretreatment of the sonicate by incubation at 65° for up to 5 min were studied.

For the arylamidase, further studies were made on the supernatant of a 27,000 g x 10 min centrifugation of crude cell homogenates. Optimum pH was determined in 0.1M HEPES buffers between pH 6.0 and pH 8.0.

Cytochemical staining of single cells for alkaline phosphatase activity was done by two methods. For visualization of activity of cells on microscopic slides, the incubation solution included 0.1M Tris-borate buffer, pH 9.0, 5mM $MgCl_2$, 1.0mM naphthol AS-BI phosphate, (Sigma Chemical Company) and 1.0mM fast blue BB salt. Cells were sedimented onto slides which were then immersed in the substrate solution for 1 hour at 37°C. The slides were then washed once with 0.2M sodium acetate buffer, pH 5.0 and covered while still wet with a glass cover slip for viewing under the microscope. For flow cytometric assay of alkaline phosphatase, 0.2mM fast red TR salt (Sigma Chemical Company, Saint Louis) was substituted for the fast blue BB salt. Higher concentration of fast red TR salt produced much non-specific fluorescence in the cells in the absence of naphthol AS-BI phosphate. Incubation times were reduced to 5 minutes also to minimize background fluorescence. Fluorescent product was quantified in single cells with a flow cytometer (40) equipped with

an argon laser tuned for excitation at 514.5 nm. Emitted light was filtered through a Corning 2-60 long pass filter which has 50% transmission at 625nm.

Cytochemical staining for the arylamidase is a modification of the previously described method (4). The staining solution contained 1 mM CBZ-ala-arg-arg-MNA, 3mM 5-nitrosalicylaldehyde, 0.25mM DTE, 0.25 mM EDTA in 0.1 M NaCl buffered with 0.05 M sodium phosphte, pH 6.0. Cells were incubated with 2.0 ml of the staining solution for 5 min at 37°C (monolayer cells were covered with 2.0 ml of the same medium), and then washed once with 5 ml of 0.2 M sodium acetate buffer, pH 5.0. Monolayer cells were covered with glass cover slips for viewing under the microscope. Photomicrographs were obtained with a Zeiss M35 camera attached to a Zeiss Universal microscope. Kodak Tri-X pan, ASA 400 film, was used for photography. Flow cytometric analysis of intact cells for either alkaline phosphatase or the arylamidase was usually done with 10^6 cells resuspended in 2 ml of 0.2 M sodium acetate, pH 5.0, following the 5 min incubation in the staining solution. Flow cytometric analysis of the arylamidase activity was performed using an argon laser with excitation at 457.9 nm and filtering the emitted light with a Corning 3-67 long pass filter which has 50% transmission at 555 nm.

RESULTS

Biochemical assay of enzyme levels.

Results of the assays for eleven hydrolytic activities in homogenates of WI-38 and VA-13 cells are shown in Table 2. In both cell types all activities increased significantly as the cells reached confluency. The arylamidase that hydrolyzes benzyloxycarbonyl-alanyl-arginyl-arginyl-4-methoxy-2-naphthylamide (CBZ-ala-arg-arg-MNA) was approximately 20 times higher in confluent WI-38 cells than in confluent VA-13 cells. Alkaline phosphatase activity as measured by hydrolysis of p-nitrophenyl phosphate was at the same level in both cell lines during exponential growth but reached a 3-fold higher level in the VA-13 cells at confluency. These two activities were characterized further.

Characterization of the alkaline phosphatase

Sonicated preparations of WI-38 cells and VA-13 cells were assayed for alkaline phosphatase in the presence of either L-phenylalanine (an inhibitor of the term placental isoenzyme) or L-homoarginine (an inhibitor of the adult liver isoenzyme) (Table 3). The alkaline phosphatase from VA-13 cells was inhibited by L-phenylalanine but was not affected by L-homoarginine, whereas the alkaline phosphatase from WI 38 cells was inhibited by L-homoarginine but not by L-phenylalamine.

The placental isoenzyme of alkaline phosphatase has also been characterized by its thermal stability (7). Sonicated suspensions of WI-38 and VA-13 cells were each compared for alkaline phosphatase activity after incubating at 65°C for brief periods. The results (Fig. 1) show that alkaline phosphatase activity from WI-38 cells was abolished

after 1 min at 65°C while the activity from the VA-13 cells was slightly enhanced even after 5 min at the elevated temperature. Thus the alkaline phosphatase from WI-38 cells is heat labile, L-homoarginine sensitive, and L-phenylalanine resistant like the adult liver isoenzyme. The alkaline phosphatase from VA-13 cells is heat stable, L-phenylalanine sensitive and L-homoarginine resistant like the placental isoenzyme.

Cytochemical staining for alkaline phosphatase and its intracellular determination by flow cytometry

Cytochemical staining for alkaline phosphatase with naphthol AS-BI-phosphate as the substrate resulted in the production of green fluorescent naphthol AS-BI which could be seen within cells mounted on microscope slides. The product was too diffusible, however, to permit accurate quantification of alkaline phosphatase in individual cells by flow cytometry. Solutions of fast red TR salt added to the incubation mixture trapped the naphthol AS-BI as a red fluorescent product which remained localized with the cells. Mixtures of WI-38 and VA-13 cells were stained with this procedure and analyzed by flow cytometry. Fluorescence histograms of cell mixtures treated in several ways prior to or during the enzyme staining reaction are shown in Fig. 2. The number of cells of each type is shown as a function of the cellular fluorescence intensity (channel number) which is proportional to alkaline phosphatase level in individual cells. When the cell mixture was incubated only in the staining solution, VA-13 cells had greater fluorescence than did WI-38 cells (Fig. 2A). When L-phenylalanine was added to the incubation mixture, the fluorescence of VA-13 cells was reduced almost to the

fluorescence of the WI-38 cells (Fig. 2B). If the cell mixture was heated to 65°C for 5 min prior to the staining reaction, the fluorescence from the WI-38 cells was reduced to background levels while VA-13 cell fluorescence remained unchanged (Fig. 2C).

Characterization of the arylamidase

The pH dependency of CBZ-ala-arg-arg-MNA hydrolysis is shown in Fig. 3. Both cell lines showed a fairly sharp optimal arylamidase activity at pH 6.5. A double reciprocal plot (21) of enzyme reaction velocities as a function of substrate concentration gave a straight line that yielded apparent Km values of approximately 0.3mM for both WI-38 and VA-13 cell homogenate arylamidase (Fig. 4).

The arylamidase was further characterized at pH 6.5 with respect to chemical activators and inhibitors (Table 4). Dialysis of the homogenates removed most of the arylamidase activity, but the activity could be restored and further enhanced by dithioerythritol. EDTA enhanced the activity of the non-dialyzed homogenates approximately 30%. p-Hydroxymercuribenzoate, a thiol protease inhibitor, completely inhibited the arylamidase activity at 0.5mM. Leupeptin, an inhibitor of cathepsin B, was the most potent inhibitor ($I_{50} = 0.2 \times 10^{-6}M$). Other proteinase inhibitors, soy bean trypsin inhibitor and diisopropylfluorophosphate, were not inhibitory at the highest concentrations tested.

Cytochemical staining for CBZ-ala-arg-arg-MNA hydrolase and its determination by flow cytometry

Cytochemical staining of cells for the arylamidase activity resulted in the accumulation of an orange fluorescent product within the WI-38 cells within 5 min, and in VA-13 cells after 20 min. The product appeared as microgranules throughout the cytoplasm of the WI-38 cells (Fig. 5A, 5B). A mixture of WI-38 and VA-13 cells were analyzed for fluorescence with the flow cytometry after cells were stained for 5 min with the substrate plus 5'-nitrosalicylaldehyde. The flow histogram (Fig. 6) shows two peaks, one around channel 20 and one around channel 200.

Proof that the peaks at high fluorescence is from WI-38 cells was obtained by cell sorting. Cells from each peak were sorted onto microscope slides with a fluorescence activated cell sorter. The slides were then heated to 65°C and the cells stained with napthol AS BI phosphate for alkaline phosphatase. Cells from the low arylamidase peak were positive for alkaline phosphatase, while cells from the high arylamidase peak were negative for heat stable alkaline phosphatase.

DISCUSSION

We have assayed eleven hydrolytic activities of WI-38 and VA-13 cells. Since our objective was detection of marker enzymes and the development of flow cytometric assays for marker enzymes in normal and and transformed cells, we have not made detailed biochemical characterizations of all of these enzymes. Rather, hydrolytic activities were screened under conditions that had been reported in the literature to be optimal for the various enzymes. This approach revealed two markers--alkaline phosphatase and an arylamidase that hydrolizes CBZ-ala-arg-arg-MNA.

The alkaline phosphatase activity not only differs quantitatively between the two cell lines, but also differs qualitatively. The transformed VA-13 cells possess an enzyme that exhibits L-phenylalanine inhibition and heat stability similar to the Regan isoenzyme (20). Alkaline phosphatase in WI-38 cells is like the normal adult liver isoenzyme in that it is heat labile and is inhibited by L-homoarginine.

Our results agree with those of Knaup *et al.* (18) who showed that in WI-38 cells alkaline phosphatase became heat stable and was shifted toward the placental enzyme during transformation. Luduena and Sussman (23), however, were unable to identify the placental isoenzyme immunologically in either WI-38 or VA-13 cells. A heat stable, L-phenylalanine sensitive phosphatase has been associated with many human tumors (7), although there are exceptions (43). The fluorescent cytochemical method for alkaline phosphatase therefore may have applicability in the identification of those human tumor cells and transformed human cells in culture that have the heat stable enzyme.

Analysis of alkaline phosphatase activity by flow cytometry has been approached using fluorescent antibodies (24), but efforts using fluorescent enzyme substrates have been hampered by the diffusibility of the naphthol AS-BI product at alkaline pH. Our observation that fast red TR coupling resulted in a product which was retained within the cells offers a means to overcome this difficulty. Furthermore, of major significance is the observation that Fast Red TR salt shifts the fluorescence of the naphthol AS-BI to the near infrared region of the spectrum, rather than quenching the fluorescence as do some diazo salts. The use of fast red TR as an azo coupling agent may prove useful for cell localization of other esterases in addition to alkaline phosphatase.

The arylamidase that hydrolyzes CBZ-ala-arg-arg-MNA is similar to cathepsin B, a thiol proteinase characterized by Otto (29), Barrett (2), and Singh and Kalnitsky (36). Like cathepsin B, this arylamidase activity has an acid pH optimum (pH 6.5), and is activated by DTE and EDTA, but inhibited by p-hydroxymercuribenzoate and by leupeptin. Singh and Kalnitzky found that this substrate is hydrolyzed rapidly by purified rabbit lung cathepsin B (36). However, we cannot rule out action by other cathepsins, notably H and L, on this substrate (16,17).

Studies on protease activity in tumor cells have focused both on activity secreted into the culture medium of cells, and, as in this study, on activity within intact cells. Both cathepsin B (30) and plasminogen activator (28,38) activity have been found to be increased in the medium of cultured tumor cells, although this is not a universal finding (25) and the picture may be complicated in VA-13 cells by the presence of a protease inhibitor (32). Studies on activity within cells

are far less numerous, but in a study by Gunn, et al., on normal and SV-40 tranformed Balb/c 3T3 cells there was a reduction in activity hydrolyzing casein in the transformed cells (10). The cathepsin B-like activity in our study was greatly reduced in the SV-40 tranformed cells, in keeping with the observations on Balb/c 3T3 cells. The technique for quantitative cytochemical staining for cathepsin B-like activity offers potential for illucidating the role of intracellular proteinases in carcinogenesis.

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TABLE I

Enzyme Assay Conditions

Enzyme	Substrate*	Buffer solution	pH	Reference
Cathepsin B	CBZ-ala-arg-arg-MNA	0.1M ammonium acetate 0.25mM DTE, 0.2mM EDTA	6.3	(2)
Dipeptidylamino-peptidase I	Pro-arg-MNA	0.1M ammonium acetate	6.0	(26)
Dipeptidylamino-peptidase II	Lys-ala-MNA	0.1M ammonium acetate	5.5	(26)
Y-Glutamyl trans-peptidase	Y-Glu-MNA	0.1M Tris, 20mM glyclglycine	8.3	(34)
Plasminogen activator	CBZ-gly-gly-arg-MNA	0.1M Tris	8.0	(14)
Aryl sulfatase	p-Nitrocatechol-sulfate	0.1M sodium acetate	5.0	(30)
Acid phosphatase	p-Nitrophenyl phosphate	0.1M sodium acetate	5.0	(1)
Alkaline phosphatase	p-Nitrophenyl phosphate	0.1M Tris, 1mM $MgCl_2$	9.0	(1)
β-Galactosidase	p-Nitrophenyl-β-D-galacto pyranoside	0.1M sodium acetate	5.0	(19)
β-D-glucuronidase	p-Nitrophenyl-β-D-glucuronic acid	0.1M sodium acetate	5.0	(15)
Leucine amino-peptidase	l-Leucine p-nitroanilide	0.1M Hepes, 0.2mM $ZnCl_2$	7.5	(34)

*All substrate solutions were 1mM.

TABLE 2

Hydrolase Activities in WI-38 and VA-13 Cell Homogenates

Synthetic substrate	Enzyme activities[a] (nmol product/min/mg protein)			
	Log phase cultures		Plateau phase cultures	
	WI-38	VA-13	WI-38	VA-13
CBZ-ala-arg-arg-MNA	1.5	0.2	9.2	0.4
CBZ-gly-gly-arg-MNA	0.02	0.02	0.05	0.05
Y-Glutamyl-MNA	0.01	0.02	0.02	0.04
Leu-MNA	0.11	0.16	0.22	0.21
Lys-ala-MNA	0.08	0.15	0.27	0.20
Pro-arg-MNA	0.38	0.51	0.60	0.68
p-Nitrocatechol sulfate	0.06	0.06	0.07	0.09
p-Nitrophenyl-β-D-galactoside	0.04	0.06	0.10	0.14
p-Nitrophenyl-β-D-glucuronide	0.05	0.05	0.07	0.07
p-Nitrophenyl phosphate, pH 5	0.26	0.32	0.60	0.71
p-Nitrophenyl phosphate, pH 9	0.06	0.06	0.08	0.26

[a] Enzyme analyses were done on duplicate culture plates analyzed in duplicate. Variations in results between duplicate plates were less than 15%.

TABLE 3

Effect of L-homoarginine and L-phenylalanine on the alkaline hydrolysis of α-naphthol phosphate by extracts of WI-38 and VA-13 cells

Addition		Percent of control activity[a] WI-38	VA-13
.002M	L-Homoarginine	61	100
.01M	L-Homoarginine	22	101
.002M	L-phenylalanine	102	47
.01M	L-phenylalanine	109	14

[a] Represents mean value from 3 experiments.

TABLE 4

Chemical activation and inhibition of CBZ-ala-arg-arg-MNA arylamidase activity in homogenates of WI-38 and VA-13 cells.

Addition or treatment	Activity (% of Control)[c] WI-38	VA-13
27,000 g supernatant	100	100
Dialyzed supernatant[a]	35	44
" " + .05mM DTE	102	90
" " + .25mM DTE	119	126
" " + 1.0mM DTE	122	134
27,000 g supernatant + 0.25mM EDTA	108	118
" " " + .25mM DTE	124	134
" " + 0.25mM p-hydroxy-mercuribenzoate	5	5
" " 0.2 μM leupeptin	1	1
" " 1 mM diisopropyl-fluorophosphate[b]	94	91
" " soy bean trypsin inhibitor (.1 mg/ml)	102	100

DTE = dithioerythritol

[a] Dialysis versus 200 volumes 0.02M sodium phosphate buffer, pH 6.0 for 16 hours.

[b] Preincubation with diisopropyl fluorophosphate for 2 hours at 25°.

[c] Mean Results from 3 determinations.

LEGENDS TO FIGURES

Figure 1: Effect of heat pretreatment on alkaline phosphatase activity in WI-38 and VA-13 cells.

Figure 2: Flow cytometric histogram of alkaline phosphatase activity in a 1:3 mixture of WI-38:VA-13 cells. The mixture contained 2.5×10^5 WI-38 cells + 7.5×10^5 VA-13 cells in a total volume of 2 ml. A. Control, no pretreatment, incubated with substrate + fast red TR salt as described in Methods. B. L-phenylalanine (0.02M) added to the incubation mixture. C. The cell mixture was preheated in sodium chloride (0.15M) plus sodium phosphate (0.005 M) at 65° for 5 min and then resuspended in the staining solution for 5 min incubation at 37°. Fluorescence channel number is proportional to amount of fluorescent product per cell.

Figure 3: Effect of pH on the hydrolysis of CBZ-ala-arg-arg-MNA in homogenates of WI-38 and VA-13 cells. Ordinate represents µmols of MNA formed during 30 min incubation. WI-38 (●), VA-13 (○) x 10.

Figure 4: Reciprocal plots of the rate of hydrolysis of CBZ-ala-arg-arg-MNA as a function of substrate concentration in soluble extracts of WI-38 and VA-13 cells. Ordinate = $(\text{nmols/min/mg protein})^{-1}$ and the abscissa = (nmol/liter^{-1}).

Figure 5: Fluorescence visualization of CBZ-ala-arg-arg-MNA arylamidase in WI-38 cells (A) and in VA-13 cells (C). Following 5 min incubation with the staining solution. 5-Nitrosalicylaldehyde was used to produce insoluble product within the cells. Phase contrast micrographs of WI-38 (B) and VA-13 (D) (400X).

Figure 6: Flow cytometric histogram of CBZ-ala-arg-arg-MNA activity (fluorescence) in a 1:3 mixture of WI-38:VA-13 cells. A = VA-13 cells, B = WI-38 cells.

Figure 1

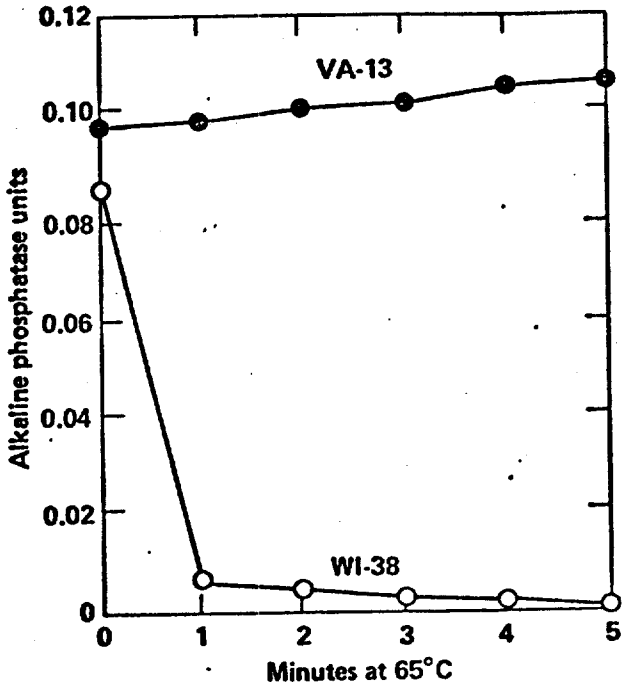

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Figure 2

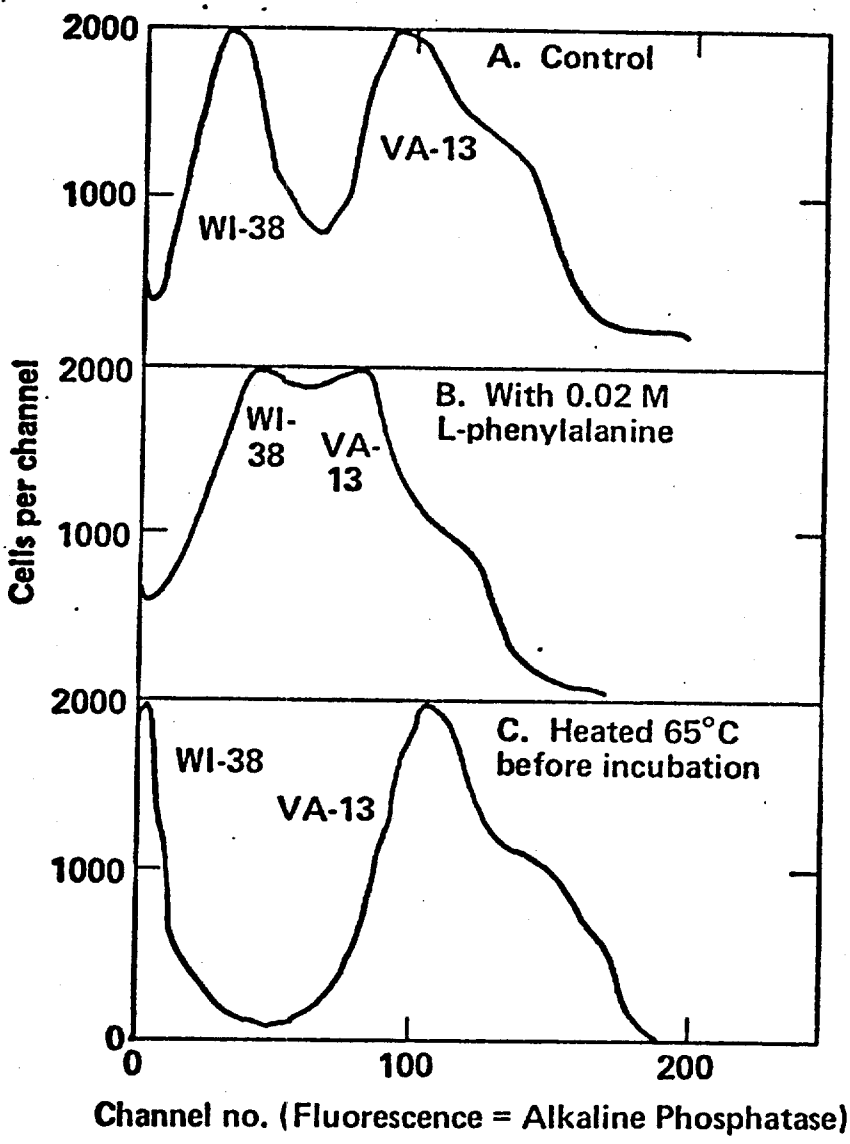

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Figure 3

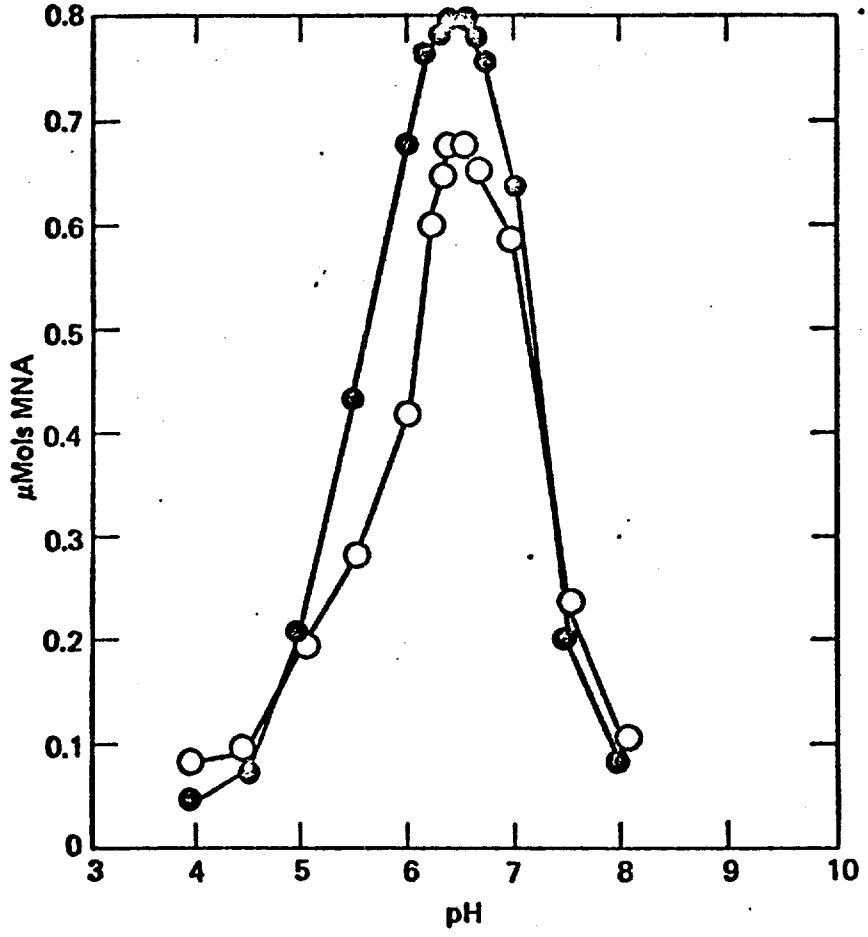

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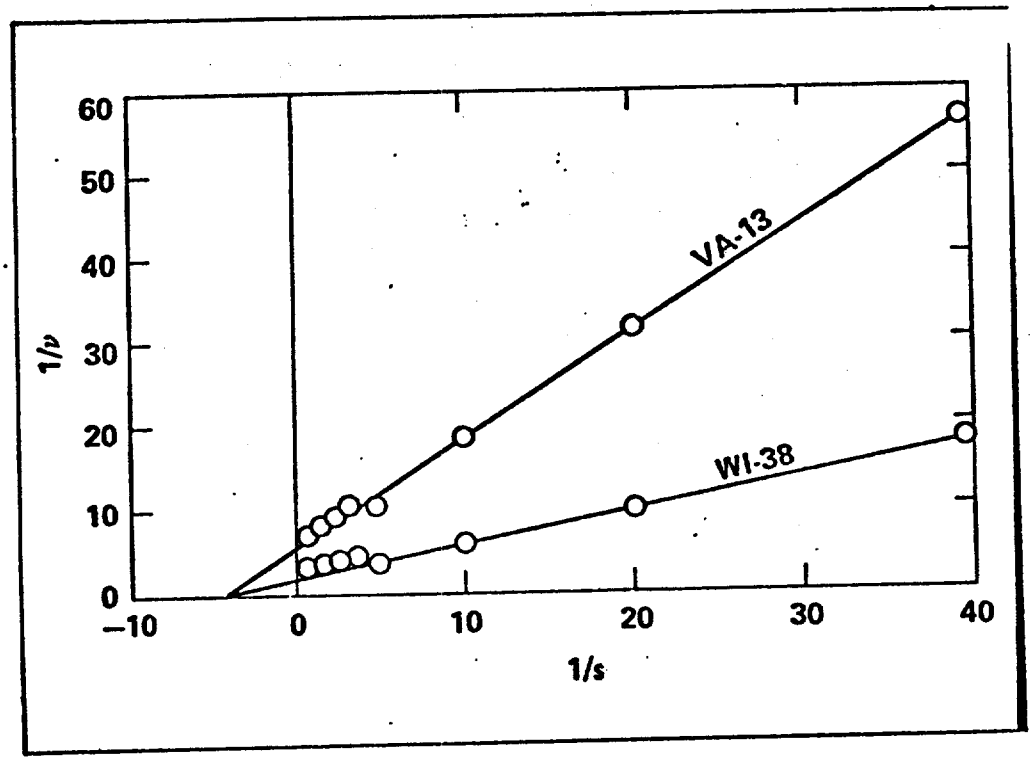
Figure 4



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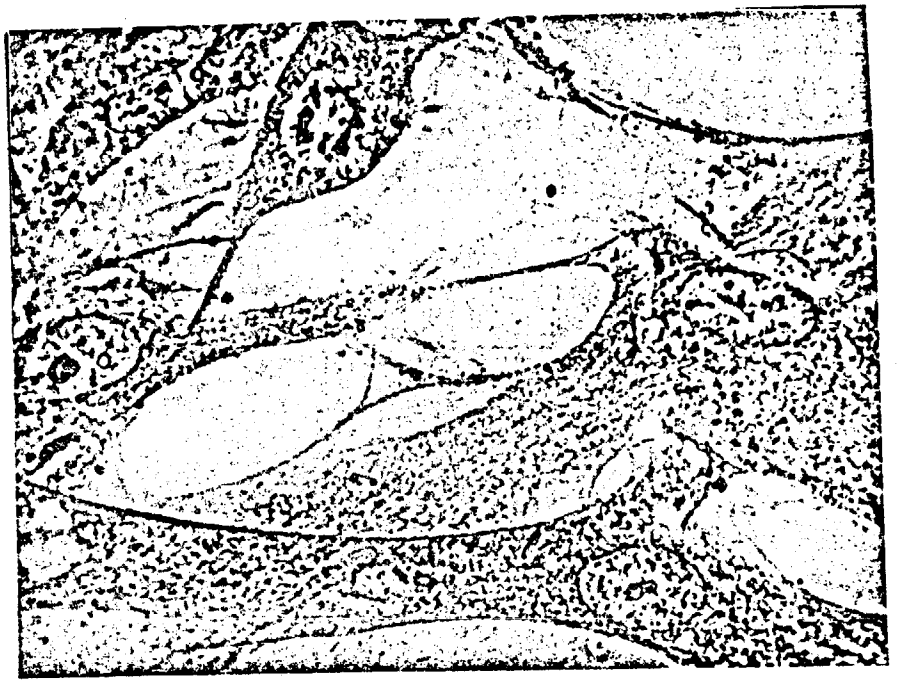
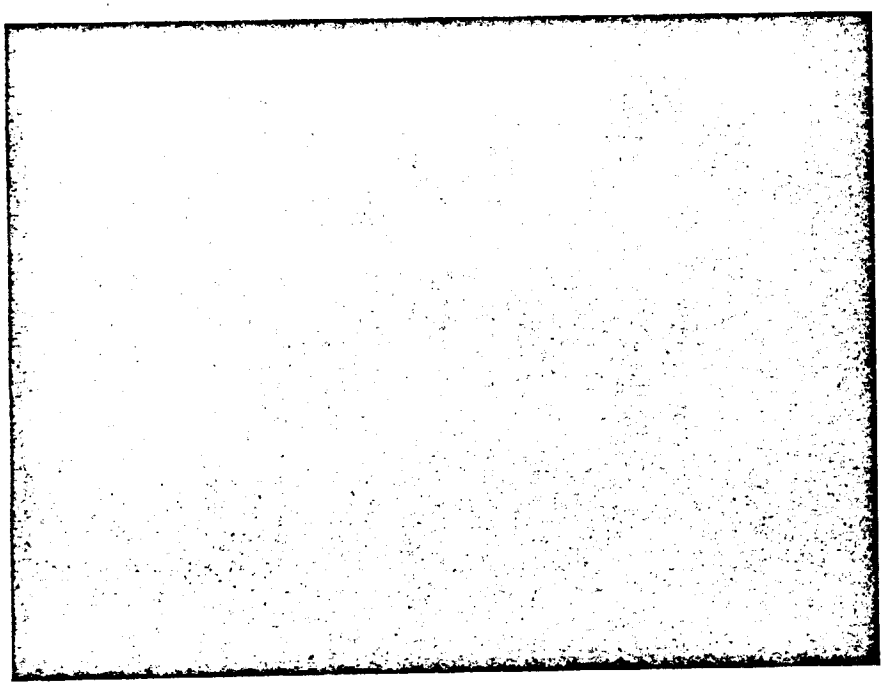
A



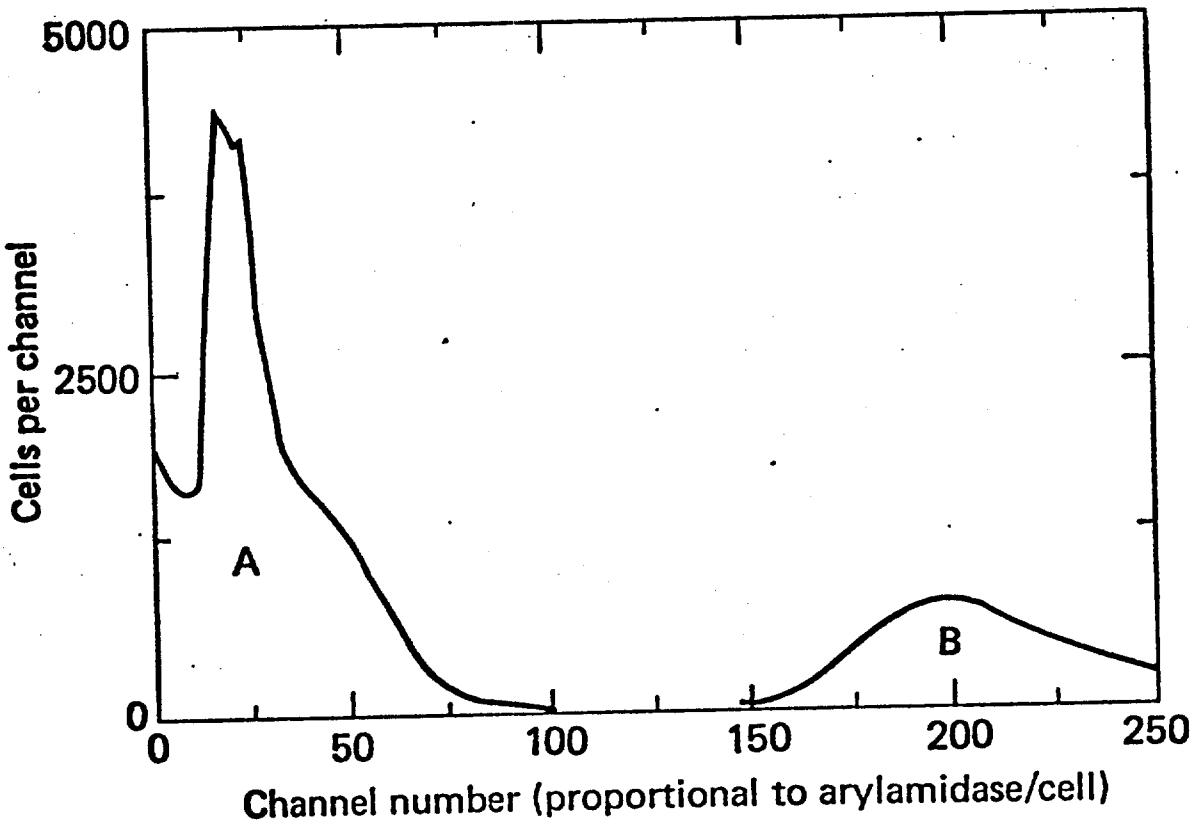
B



C

D

Figure 6



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